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The prognostic significance and impact of the CXCR4–CXCR7–CXCL12 axis in primary cutaneous melanoma*

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Summary

Background Expression of the chemokine receptor CXCR4 is known to regulate melanoma metastasis to distant sites with high expression of the CXCL12 ligand. However, the prognostic impact of CXCR4 expression and potential for autocrine-mediated activation of prosurvival mitogen-activated protein kinase signalling remains enigmatic. Furthermore, expression of the decoy receptor CXCR7 within the local cutaneous melanoma microenvironment remains undefined.

Objectives To define the contribution and prognostic impact of CXCR4–CXCR7–CXCL12 signalling in primary cutaneous melanomas and the immediate tumour microenvironment.

Methods Immunohistochemical/immunofluorescent expression of CXCR4, CXCR7 or CXC12 was analysed in human metastatic melanoma cell lines, primary cutaneous cell types and a retrospective cohort of primary melanomas/benign naevi. CXCL12 secretion by melanoma/cutaneous cells was evaluated by enzyme-linked immunosorbent assay, and autocrine CXCR4–CXCL12 signalling was investigated by addition of a CXCL12-neutralizing antibody.

Results CXCR4 expression was significantly higher in primary melanomas that subsequently metastasized after 7 years ($P = 0{\cdot}037$). Stratification for American Joint Committee on Cancer (AJCC) stage II disease revealed significantly decreased disease-free survival in patients with > 50% CXCR4 expression ($P = 0{\cdot}036$), while comparative analysis of CXCL12 expression in the adjacent epidermis of all AJCC stage melanomas revealed increased CXCL12 correlated with prolonged time to metastasis ($P = 0{\cdot}014$). CXCR7 was expressed within the primary melanoma microenvironment but was absent on primary tumours. Addition of anti-CXCL12 to BRAF-mutant melanoma cells resulted in downregulation of phospho-CXCR4 and phospho-extracellular signal-related kinase, indicating autocrine CXCR4–CXCL12 signalling.

Conclusions CXCR4 expression defines a potential prognostic biomarker for AJCC stage II melanoma. Moreover, targeting the CXCR4–CXCR7–CXCL12 axis may represent a novel therapeutic strategy to prevent early melanoma progression.

What's already known about this topic?

- CXCR4 expression is associated with poor prognostic indicators including tumour ulceration, thickness and metastasis.
- Expression of CXCR4 is crucial in regulating melanoma chemotaxis to distant secondary sites of metastasis with high expression of the ligand, CXCL12.
- Previous studies suggest a lack of correlation between melanoma CXCL12 expression and disease progression or BRAF mutational status.
- CXCR7 is an atypical/decoy CXCL12 receptor that may act to shape CXCL12 gradients.

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What does this study add?

- Elevated CXCR4 expression is a prognostic biomarker for AJCC stage II primary melanomas.
- Autocrine melanoma CXCR4–CXCL12 signalling promotes hyperactivation of prosurvival MAPK signalling.
- CXCL12 expression by stromal cells in the primary melanoma microenvironment protects from metastasis potentially by retaining tumour cells within the cutaneous microenvironment.
- Downregulation of melanoma CXCL12 is associated with an aggressive BRAF/NRAS mutant phenotype.
- CXCR7 expression by cutaneous vascular endothelium and not primary melanomas suggests a mechanism mediating tumour intravasation.

What is the translational message?

- CXCR4 expression defines a potential candidate biomarker for melanoma providing additional prognostic information to AJCC disease staging and a means of identifying high-risk patient subgroups.
- The reported role of CXCR4–CXCR7–CXCL12 chemokine signalling within the primary cutaneous melanoma microenvironment highlights the potential for targeting this chemokine axis to prevent the development of metastatic disease.

Cutaneous melanoma is the most aggressive form of skin cancer, with an increasing incidence worldwide. This is particularly so in younger individuals, for whom death from metastatic disease accounts for 80% of all skin cancer deaths. Moreover, despite recent advances in targeted therapies to oncogenic BRAF/NRAS-induced prosurvival mitogen-activated protein kinase (MAPK) signalling and novel immunotherapies, there are still no consistently beneficial treatments for cutaneous metastatic melanoma or reliable biomarkers able to predict disease progression for those patients diagnosed with seemingly low-risk, early American Joint Committee on Cancer (AJCC) stage primary tumours.[1,2] An urgent and improved understanding of the primary tumour microenvironment is therefore required in order to identify credible prognostic biomarkers able to predict those primary melanomas at greatest risk of disease progression, and to inform the development of more efficacious precision-based therapeutic strategies to prevent tumour migration and metastasis.

Chemokines and their receptors play a diverse and critical role in normal cellular homeostasis. Principally the chemokine system induces the directional migration of chemokine receptor-expressing cells down chemokine ligand gradients, with involvement in processes as early as embryogenesis and in normal immune responses.[3] Unsurprisingly, cancer cells may hijack chemokine axes, upregulate chemokine receptors or secrete chemokine ligands, directly impacting on the behaviour of the tumour cell itself, the tumour microenvironment and establishment of metastasis. In this context deregulation of CXCR4–CXCR7–CXCL12 signalling is a well-described mechanism of tumour cell migration and metastasis.[4]

The gain of CXCR4 expression by melanoma cells not only permits migration towards CXCL12 at common metastatic sites, but may also promote tumour cell survival and proliferation through paracrine/autocrine-mediated activation of MAPK cell signalling, highlighting a potential role for CXCR4–CXCL12 signalling in melanomagenesis.[5,6] Furthermore, elevated CXCR4 expression has been linked to melanoma ulceration and thickness and disease progression,[7–10] although to date CXCR4 expression as a prognostic biomarker remains ambivalent.[11] Similarly, the role of the CXCL12 chemokine ligand within primary cutaneous melanomas has largely been overlooked. Importantly, CXCL12 functions to shape the primary tumour infiltrate, promoting angiogenesis by recruiting potent proangiogenic plasmacytoid dendritic cells or creating an immunosuppressive tumour microenvironment through recruitment of $CD4^+$ T regulatory cells.[12] However the effect of CXCL12 within the local microenvironment of primary cutaneous melanoma remains unexplored, with only one study reporting an apparent correlation between the absence of tumoral expression and poor prognosis.[11]

To add complexity to CXCR4–CXCL12 signalling, recent studies have identified CXCR7 as a chemokine receptor that binds to CXCL12 with 10-fold greater affinity compared with CXCR4.[13] However, despite its classical features as a chemokine receptor, in general CXCR7 functions as a 'decoy' receptor that 'scavenges' and hence removes monomeric CXCL12 to dampen or inhibit CXCR4–CXCL12 signalling. CXCR7 also has

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the ability to heterodimerize with CXCR4, thereby potentially modulating downstream signalling including proliferation, survival and cell migration. Indeed, the upregulation of CXCR7 has been shown to promote growth and migration of a number of tumour types[14–16]; however, to date there are no reports of CXCR7 expression or function in melanoma.

Although it is known to influence the metastasis of melanoma to distant sites, the role of CXCR4–CXCR7–CXCL12 signalling in the primary melanoma local environment and its influence on early tumour migration and invasion remain unclear.[5]

In the present study we report high levels of CXCR4 expression within primary melanomas as a novel prognostic biomarker, identifying high-risk AJCC stage II tumours. We further identify and characterize CXCL12 and CXCR7 expression within primary melanomas and the cutaneous microenvironment, highlighting a link between relative CXCL12 expression and BRAF/NRAS mutational status and associated autocrine signalling, and a mechanism by which the CXCR4–CXCR7–CXCL12 axis promotes melanoma migration and metastasis.

Materials and methods

Patient demographics

Retrospective formalin-fixed paraffin-embedded (FFPE) tissue was obtained from a cohort of 77 samples. These comprised 13 benign melanocytic naevi and 64 primary AJCC stage I, II or III melanomas at the time of diagnosis and following excision at the Royal Victoria Infirmary, Newcastle upon Tyne between January 2003 and May 2005. Full ethical permission was obtained (Newcastle and North Tyneside research ethics committee, reference 08/H0906/95+5), and all aspects of the study were performed in accordance with the Declaration of Helsinki principles. Data were correlated with clinical outcome after a minimum of 7 years of follow-up, allowing the correlation of CXCR4 expression in the primary tissue sample with the time to development of metastatic disease (disease-free survival). Disease recurrence was defined by the time to first radiological or tissue diagnosis of metastatic disease (nodal or systemic) from the point of initial primary tumour excision, or time to death from melanoma (melanoma-specific mortality). Reporting of the data was performed in line with the REMARK guidelines for prognostic biomarkers.[17]

Growth and maintenance of melanoma cell lines and primary skin cells

Human metastatic melanoma cell lines CHL-1 (BRAF/NRAS wild-type), A375, WM-164 (both BRAF V600E mutated) and WM1361 (NRAS mutant), and EA.hy926 endothelial cell lines were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). They were cultured as previously described,[18,19] with authenticity of melanoma cell lines confirmed by melan-A expression analysis[20] and using custom TaqMan single-nucleotide polymorphism genotyping assays (Applied Biosystems, Warrington, U.K.) to verify BRAF/NRAS mutational status.[21] Primary human epidermal keratinocytes, dermal fibroblasts and melanocytes were isolated from redundant foreskin,[22] breast or abdominal skin. Primary cultures were expanded in human keratinocyte growth supplement EpiLife medium (Invitrogen, Paisley, U.K.), Dulbecco's Modified Eagle's Medium (Lonza, Vervies, Belgium) or melanocyte 254 medium (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). Full ethical permission for the isolation of primary cells was obtained as described above.

Immunohistochemistry and immunofluorescence

Immunohistochemistry for the expression of CXCR4 and CXCR7 expression in FFPE sections of naevi, primary melanomas or control tonsil was carried out as previously described.[23] CXCR4 was detected with monoclonal anti-CXCR4 (clone 44716, 2·5 μg mL^{-1} with antigen retrieval performed using 0·1 mol L^{-1} Tris-HCl pH 7·6; R&D Systems Europe, Abingdon, U.K.) or CXCR7 with monoclonal anti-CXCR7 (clone 11GB, 5 μg mL^{-1} with antigen retrieval performed using 0·1 mol L^{-1} Tris-HCl pH 9; Chemocentryx, San Francisco, CA, U.S.A.) overnight at 4 °C, as previously described.[23] Isotype-matched negative control antibodies were included during all staining procedures to demonstrate antibody specificity. Images were acquired by light microscopy with a ×20 objective (Zeiss Axio Imager; Carl Zeiss Microscopy, New York, NY, U.S.A.).

Immunofluorescence for the detection of CXCR7 and CXCL12 was carried out on metastatic melanoma and EA.hy926 cell lines, primary melanocytes, keratinocytes or dermal fibroblasts seeded at 2×10^5 cells per well for 24 h on glass coverslips, prior to fixation in ice-cold 4% paraformaldehyde and permeabilization with 0·2% Triton X-100. Immunolabelling of cells was performed with monoclonal anti-CXCR7 (clone 11GB, 10 μg mL^{-1}; Chemocentryx) or polyclonal anti-CXCL12 (clone 9797, 1 μg mL^{-1}; Abcam, Cambridge, U.K.) overnight at 4 °C and detected with Oregon green 488 conjugated to antimouse or antirabbit IgG (Thermo Fisher Scientific Inc.). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific Inc.).

Fluorescent double staining of primary FFPE melanoma sections with CXCL12 and melan-A (control melanocyte marker) was performed as previously described.[5] Antigen retrieval was performed in 10 mmol L^{-1} sodium citrate (pH 6) for 1 min and primary CXCL12 was detected with rabbit anti-CXCL12 (clone 9797, 1 μg mL^{-1}; Abcam), or for melan-A expression with mouse antimelan-A (clone 731, 0·4 μg mL^{-1}; Abcam). Primary antibody binding was detected with Alexa Fluor® 568 conjugated to antirabbit IgG (clone 175471; Abcam) or Oregon green 488 conjugated to antimouse IgG (Thermo Fisher Scientific Inc.), and the nuclei were counterstained with DAPI 1 μg mL^{-1}. Tumour sections or stained cells were finally mounted onto glass coverslips with fluorescence mounting medium (Vector Laboratories Inc., Burlingame, CA, U.S.A.),

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and the images captured by confocal microscopy using ×40 or ×20 objectives (Leica TSC SP2 UV; Leica Microsystems GmbH, Heidelberg, Germany).

Quantification of immunohistochemical CXCR4 and immunofluorescent CXCL12 expression

The mean percentage immunohistochemical expression of CXCR4 positively stained cells was derived by the analysis of up to 10 representative fields of vision using Leica QWin image analysis software (Leica Microsystems GmbH) as previously described.[23] CXCL12 fluorescence per tumour cell (relative to melan-A) or within the epidermis of melanoma tissue sections (relative to DAPI) was quantified above background fluorescence from four or two representative images per tumour, respectively, using ImageJ software (http://imagej.net).

Western blotting

Preparation of whole-cell lysates and Western blotting for anti-CXCR4 (clone 2074, 0·1 μg mL^{-1}; Abcam), antiphospho-CXCR4 (clone 74012, 1 : 1000; Abcam) or phospho-extracellular signal-related kinase (ERK) (clone 43775, 1 : 5000; Cell Signalling Technology, Danvers, MA, U.S.A.) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (loading control) (clone 2118, 1 : 5000; Cell Signalling Technology) was performed as described previously.[18]

Enzyme-linked immunosorbent assay for CXCL12

Human melanoma cell lines were seeded at a density of 5×10^4 cells per well in six-well plates for 72 h prior to the analysis of CXCL12 in cell supernatants using a commercially available enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Human CXCL12/SDF-1 alpha Quantikine ELISA Kit, DSA00; R&D Systems Europe).

Treatment of cells with CXCL12-neutralizing antibody

WM-164 melanoma cells were seeded at $1{\cdot}5 \times 10^5$ cells per well in six-well tissue culture plates 24 h prior to the addition of 1 μg mL^{-1} anti-CXCL12 neutralizing antibody (clone 9797; Abcam) or IgG isotype control antibody (clone 171870; Abcam) for 5–30 min before harvesting cell lysates for Western blotting.

Statistics

CXCR4 immunohistochemical data were expressed as median expression levels between groups. Independent group analysis between localized and metastatic disease (eventual AJCC stage I/II vs. eventual AJCC stage III/IV disease) was determined by Mann–Whitney U-test. The difference between 'high' and 'low' CXCR4 expression levels for survival curve analysis was tested using the Wilcoxon signed-rank test. Univariate and subsequent log-rank (Mantel–Cox) survival analysis were undertaken using R 2.15.0 (https://www.r-project.org/). The Mann–Whitney U-test was used for analysis of CXCL12 fluorescence between localized and metastatic disease (eventual AJCC stage I/II vs. eventual AJCC stage III/IV disease) or BRAF-mutated and wild-type melanomas, while Pearson product moment correlation analysis was used to correlate mean epidermal CXCL12 fluorescence with time to metastasis or cytoplasmic CXCR4 expression. Relative densitometry of phospho-CXCR4:GAPDH or phospho-ERK:GAPDH was analysed by one-way ANOVA with Dunnett's post hoc correction. A P value $< 0{\cdot}050$ was considered significant throughout. All data were analysed using the statistical software Prism 5 (GraphPad Software, La Jolla, CA, U.S.A.).

Results

CXCR4 expression is a prognostic biomarker in cutaneous melanoma

To assess CXCR4 expression in the context of melanoma, CXCR4 expression levels were initially compared *in vitro* in a panel of BRAF/NRAS wild-type or mutated cutaneous melanoma cell lines with expression in primary cutaneous melanocytes. Western blot analysis of total CXCR4 expression revealed variable but consistent expression of CXCR4 in primary melanocytes, as well as in cutaneous CHL-1 (BRAF/NRAS wild-type), WM-164 (BRAF V600E mutated) and WM-1361 (NRAS mutated) metastatic melanoma cell lines (Fig. 1a,b), but with enhanced expression observed in all melanoma cell lines compared with expression in normal melanocytes.

Next, to determine the potential for CXCR4 expression as a prognostic biomarker for melanoma *in vivo*, and as a means of identifying high-risk tumours at diagnosis, CXCR4 expression levels were evaluated in a cohort of primary melanomas of differing AJCC disease stage. Primary melanomas showed variable expression of both cytoplasmic and nuclear CXCR4 (Fig. 1c). Comparison of total CXCR4 expression levels in melanomas that remained localized with expression in tumours that subsequently metastasized after 7 years of follow-up (eventual AJCC stages I/II vs. stages III/IV) revealed significantly higher median CXCR4 expression in the metastatic cohort (56·1% vs. 37·58%, respectively; Mann–Whitney U-test, $P = 0{\cdot}037$) (Fig. 1d). This highlights an association between CXCR4 upregulation and the development of metastatic disease, and is consistent with the reported role of CXCR4 in cancer cell migration.[5]

A Wilcoxon signed-rank test confirmed a differential cut-off point of 50% CXCR4 expression as appropriate for the performance of survival-curve analysis, where 'high CXCR4' expression was defined as > 50%, and 'low CXCR4' expression as < 50%. Univariate analysis of mean percentage total CXCR4 expression in all AJCC stage melanomas revealed a nonsignificant decrease in disease-free survival from 65·5% for individuals expressing low CXCR4 (< 50%) to 47·6% in individuals expressing high CXCR4 (> 50%) over 7 years: log-rank (Mantel–Cox) test, hazard ratio (HR) 1·99, 95% confidence interval

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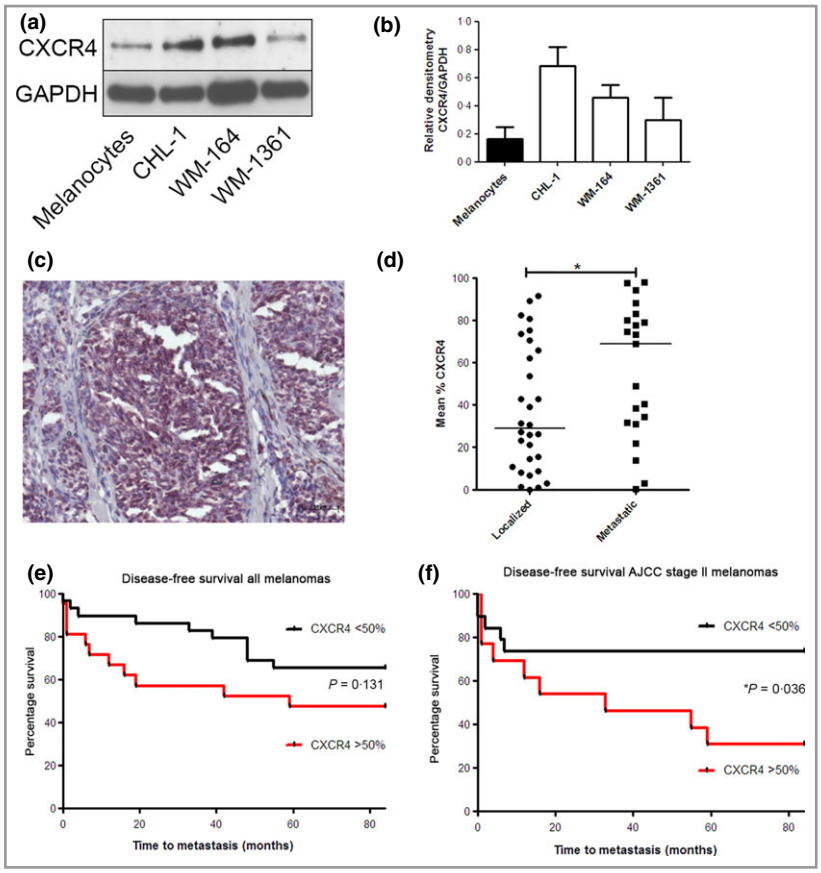

Fig 1. CXCR4 expression is a prognostic biomarker in cutaneous melanoma. (a) Representative Western blot of CXCR4 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading control expression in CHL-1 (wild-type), WM-164 (BRAF V600E mutated) or WM-1361 (NRAS mutated) human metastatic cutaneous melanoma cell lines or primary melanocytes (n = 4). (b) Densitometric analysis of CXCR4 relative to GADPH expression. Each bar represents the mean of four replicates of CXCR4 band intensity normalized to GAPDH band intensity (CXCR4/GAPDH) for each cell line or primary melanocytes derived from four independent donors, and is expressed relative to the mean of each individual experiment (mean ± SD, n = 4). (c) Representative immunohistochemical expression of CXCR4 in a primary American Joint Committee on Cancer (AJCC) stage III cutaneous melanoma depicting both nuclear and cytoplasmic CXCR4 expression. Images were acquired by light microscopy with magnification ×20, scale bar = 50 μm. (d) Scatter graph representing the mean percentage of positive CXCR4-staining cells in localized (eventual AJCC stage of disease I/II) and metastatic melanomas (eventual AJCC stage III/IV) after 7 years of follow-up. Horizontal bars represent the median staining percentage (Mann–Whitney U-test, P = 0·037). (e) Univariate analysis of mean percentage CXCR4 expression and disease-free survival (7 years) in all AJCC stage melanomas. Vertical lines represent individual patients developing a metastasis. Log-rank (Mantel–Cox) test, hazard ratio 1·99 (95% confidence interval 0·81–4·91), P = 0·13. (f) Univariate analysis of percentage CXCR4 expression and disease-free survival (7 years) in AJCC stage II melanomas. A significantly increased risk of metastasis was detected in AJCC stage II tumours expressing > 50% mean CXCR4 compared with tumours expressing < 50% mean CXCR4. Vertical lines represent individual patients developing a metastasis. Log-rank (Mantel–Cox) test, hazard ratio 3·24 (95% confidence interval 1·08–9·73), P = 0·036.

(CI) 0·81–4·91, P = 0·13; Figure 1e. However, stratification for AJCC stage II melanomas at diagnosis revealed that high CXCR4 expression (> 50%) was associated with significantly decreased disease-free survival (reduced to 30·8%, compared with 73·7% for individuals with low, < 50%, CXCR4 expression) and patients who remained disease free over 7 years: log-rank (Mantel–Cox) test, HR 3·24, 95% CI: 1·08–9·73, P = 0·036; Figure 1f. Collectively, these results highlight a threefold increased risk of disease reoccurrence in individuals with high total CXCR4 expression (> 50%) (Fig. 1f), further suggesting high CXCR4 expression as a prognostic biomarker for AJCC stage II melanomas.

Expression and secretion of CXCL12 in cutaneous melanoma cell lines and primary cutaneous cells

To assess the role of CXCL12 in the primary tumour environment of cutaneous melanoma, initial immunofluorescence studies for expression of CXCL12 were performed in melanoma cell lines, as well as in primary cells known to be present within the local tumour microenvironment, including primary melanocytes, keratinocytes and dermal fibroblasts.

Immunofluorescent staining for CXCL12 revealed variable cytoplasmic expression by all cell lines and primary cells, indicating that under normal and unstimulated cell-culture

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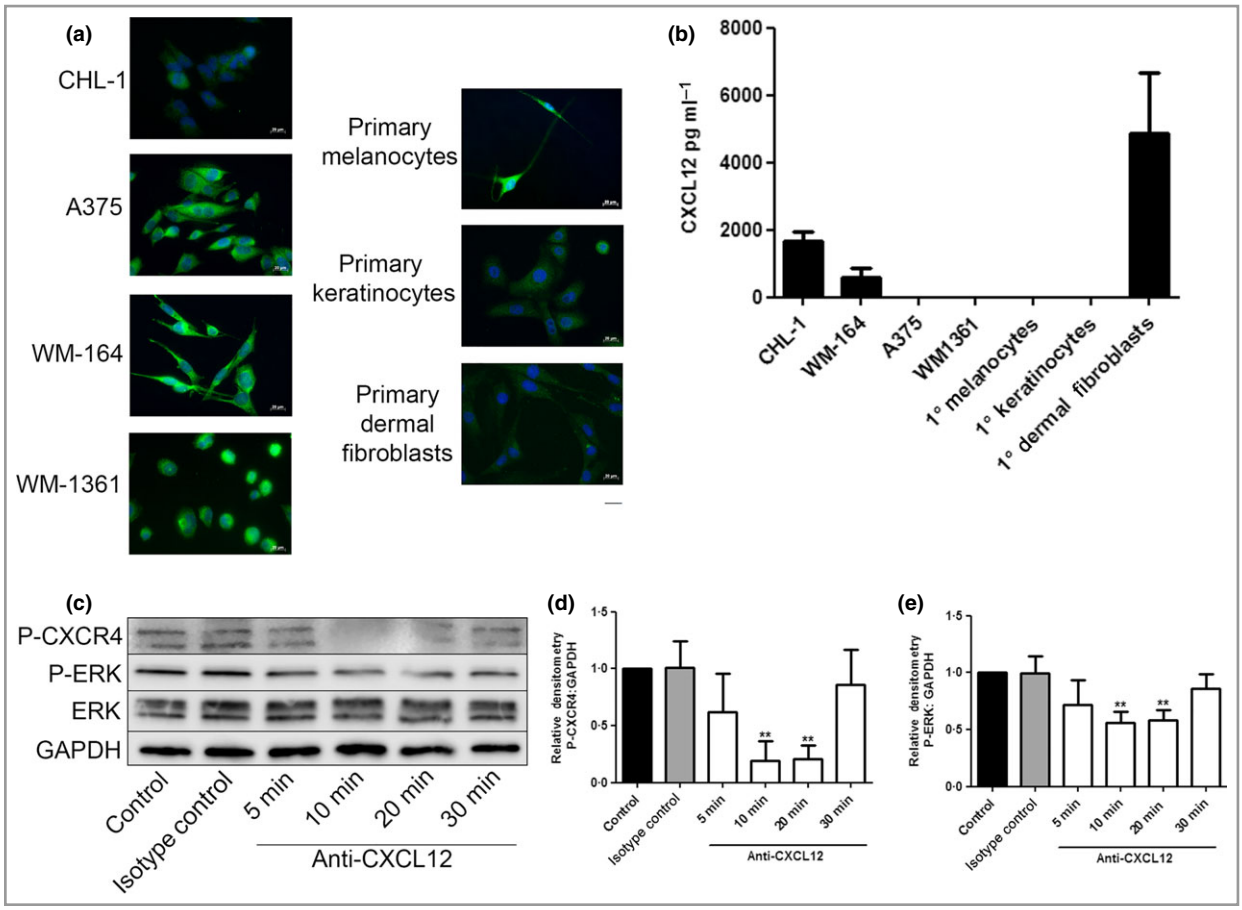

Fig 2. Expression and secretion of CXCL12 in cutaneous melanoma cell lines and primary cutaneous cells. (a) Representative image from three replicate experiments for the immunofluorescent expression of CXCL12 in cutaneous metastatic melanoma cell lines (CHL-1, A375, WM-164), primary melanocytes, keratinocytes and dermal fibroblasts. Images were acquired with negative antibody control set to no detectable fluorescence. Green depicts CXCL12 positivity and blue shows 4′,6-diamidino-2-phenylindole nuclear staining. Images were acquired by confocal microscopy with magnification ×40, scale bar = 20 μm. (b) CXCL12 (pg mL^{-1}) secretion by human metastatic cutaneous melanoma cell lines (CHL-1, WM-164, A375, WM-1361) and primary melanocytes, keratinocytes and dermal fibroblasts. Each bar represents the mean ± SD CXCL12 secretion of three independent experiments of cell lines or from primary cutaneous cells derived from four independent donors. (c) Representative Western blot of phospho-CXCR4 (P-CXCR4), phospho-extracellular signal-related kinase (P-ERK), total ERK and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading control expression in WM-164 (BRAF V600E mutated) metastatic melanoma cells treated with anti-CXCL12 neutralizing antibody or IgG isotype control for 5, 10, 20 or 30 min; n = 3. (d) Densitometric analysis of P-CXCR4 relative to GADPH expression. Each bar represents the mean of three replicates of P-CXCR4 band intensity normalized to GAPDH band intensity (P-CXCR4 : GAPDH), and expressed relative to the mean of each individual experiment (mean ± SD, n = 3). Statistics were acquired by one-way ANOVA with Dunnet's post hoc correction, **P = 0·001. (e) Densitometric analysis of P-ERK relative to GADPH expression. Each bar represents the mean of three replicates of P-ERK band intensity normalized to GAPDH band intensity (P-ERK:GAPDH), and expressed relative to the mean of each individual experiment (mean ± SD, n = 3). Statistics acquired by one-way ANOVA with Dunnet's post hoc correction, **P = 0·003.

conditions, CXCL12 is likely produced *in situ* (Fig. 2a). To investigate CXCL12 secretion by differing cutaneous cell types, CXCL12 was quantified in cell-culture supernatants derived from confluent cutaneous melanoma cell lines, primary melanocytes, keratinocytes and fibroblasts using a commercial ELISA assay (Fig. 2b). Surprisingly, although all cells displayed cytoplasmic expression of CXCL12, only CHL-1 (BRAF/NRAS wild-type) and WM-164 (BRAF V600E mutated) melanoma cells and dermal fibroblasts secreted detectable levels of CXCL12 (limit of detection 156 pg mL^{-1}; Fig. 2b).

To investigate the possibility of CXCR4–CXCL12 autocrine cell signalling and the impact on MAPK cell signalling, a neutralizing antibody to CXCL12 or isotype control antibody was added to WM-164 cells. Western blotting revealed a significant reduction of phospho-CXCR4 expression after treatment with CXCL12-neutralizing antibody for 10 or 20 min (Fig. 2c,d; one-way ANOVA with Dunnett's post hoc correction, P = 0·001). This was accompanied by significant inhibition of MAPK cell signalling and a concomitant reduction in the expression of

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phospho-ERK (Fig. 2c,e; one-way ANOVA with Dunnet's post hoc correction, $P = 0{\cdot}003$).

Downregulation of melanoma CXCL12 is associated with an aggressive BRAF/NRAS mutant phenotype

To investigate further the impact of CXCL12 autocrine signalling in cutaneous melanomas *in situ* and to determine the expression of CXCL12 in the immediate tumour microenvironment, CXCL12 expression was evaluated in a cohort of 24 melanocytic naevi or primary melanomas of differeing AJCC disease stage with double staining for melan-A, used as a melanocytic marker (Fig. 3a).

In line with previous observations, CXCL12 expression was detected within the dermis, localized to dermal fibroblasts,[24] and interestingly was also strongly expressed by stromal cells within the epidermis adjacent to all primary melanomas (Fig. 4a). In contrast, tumoral CXCL12 in all AJCC stage primary cutaneous melanomas colocalized with melan-A expression (Fig. 3a).

Analysis of CXCL12 within primary melanomas revealed consistent expression of CXCL12 by tumour cells, albeit at low levels. However, expression did not vary significantly between localized or metastatic melanomas (Fig. 3b; Mann–Whitney U-test; $P = 0{\cdot}11$). Remarkably, comparison of CXCL12 expression with NRAS/BRAF mutational status revealed significantly reduced CXCL12 expression in BRAF/NRAS-mutant tumours compared with expression in wild-type tumours (Fig. 3c; Student's t-test; $P = 0{\cdot}032$), suggesting that the downregulation of CXCL12 secretion may be mediated by activation of MAPK signalling.

Increase of CXCL12 in primary cutaneous melanoma epidermis correlates with increased time to metastasis

To explore CXCL12 expression within the epidermis of primary melanomas and any association with disease progression, CXCL12 fluorescence was also quantified in the peritumoral adjacent epidermis of all primary melanomas within the study cohort. The results revealed consistent expression of epidermal CXCL12, in particular by keratinocytes in the stratum corneum (Fig. 4a) in the adjacent epidermis overlying all primary melanomas (Fig. 4b). Comparative analysis of epidermal CXCL12 expression in the adjacent epidermis of all AJCC stage melanomas vs. tumour progression further revealed a significant correlation between increased CXCL12 expression and increased time to metastasis (Fig. 4c; Pearson correlation $P = 0{\cdot}013$). This suggests that CXCL12 within the immediate tumour microenvironment limits disease metastasis. Furthermore, a significant correlation of adjacent epidermal CXCL12 expression with tumoral expression of cytoplasmic CXCR4 was also observed (Fig. 4d; Pearson correlation $P = 0{\cdot}009$). This highlights a possible relationship between CXCL12 expression in the microenvironment and chemokine receptor activation in the tumour, and suggests a previously undescribed intimate relationship between microenvironmental CXCL12, melanoma phenotype and clinical outcome.

Expression of CXCR7 in cutaneous melanoma and the primary tumour microenvironment

There is a paucity of studies exploring the role of CXCR7 in melanoma biology and within the immediate cutaneous microenvironment, given its potential effect on the CXCR4–CXCL12 axis. To address the potential impact of CXCR7 on this chemokine–receptor axis, immunofluorescent analysis for the expression of CXCR7 was performed in CHL-1 or WM-164 melanoma cell lines, or in primary melanocytes. Expression levels were compared with known CXCR7 expression levels in control EA.hy926 endothelial cells (Fig. 5a). While cytoplasmic CXCR7 was clearly expressed by EA.hy926 cells, there was no apparent expression of CXCR7 by either cutaneous melanoma cell lines or by primary melanocytes (Fig. 5a).

To extend these *in vitro* findings, CXCR7 expression was determined in the study patient cohort, where the results reflected the observed *in vitro* expression pattern. No apparent expression of CXCR7 was evident in any primary melanoma or benign naevus. Intriguingly, expression of CXCR7 was clearly detectable within the tumour microenvironment of the dermis of seven primary melanomas; specifically it was detected on cells lining the lumen of blood vessels and capillaries (Fig. 5b). However, these observations did not correlate with either AJCC disease stage or with BRAF/NRAS mutational status (data not shown). Collectively these data suggest that CXCR7 expressed by the cutaneous vascular endothelium may act as a decoy receptor for CXCL12, modulating chemokine gradients within the tumour microenvironment.

Discussion

Tumour thickness and ulceration are two components of the AJCC melanoma staging system that currently provide the most important prognostic information for patients diagnosed with primary cutaneous melanomas.[25] However, neither these nor any other prognostic indicators are reliably able to identify 'high-risk' patients, whose tumours will eventually progress, emphasizing the acute demand for credible, novel biomarkers with predictive accuracy of metastatic progression.

Current evidence suggests that chemokines and their receptors play a critical role in many stages of cancer progression, with the CXCR4–CXCL12 axis known to be key to melanoma metastasis to distant secondary sites.[4] However, despite reports demonstrating an association of CXCR4 expression with adverse clinical features,[7–10] to date no studies have characterized the prognostic significance of CXCR4 expression in well-powered melanoma cohorts. In contrast to previous studies, reporting an apparent correlation of CXCR4 expression with melanoma ulceration, increased thickness, metastasis and morbidity,[7–10] Mitchell *et al.* suggested that CXCR4 expression correlates with better prognostic features, such as the absence of

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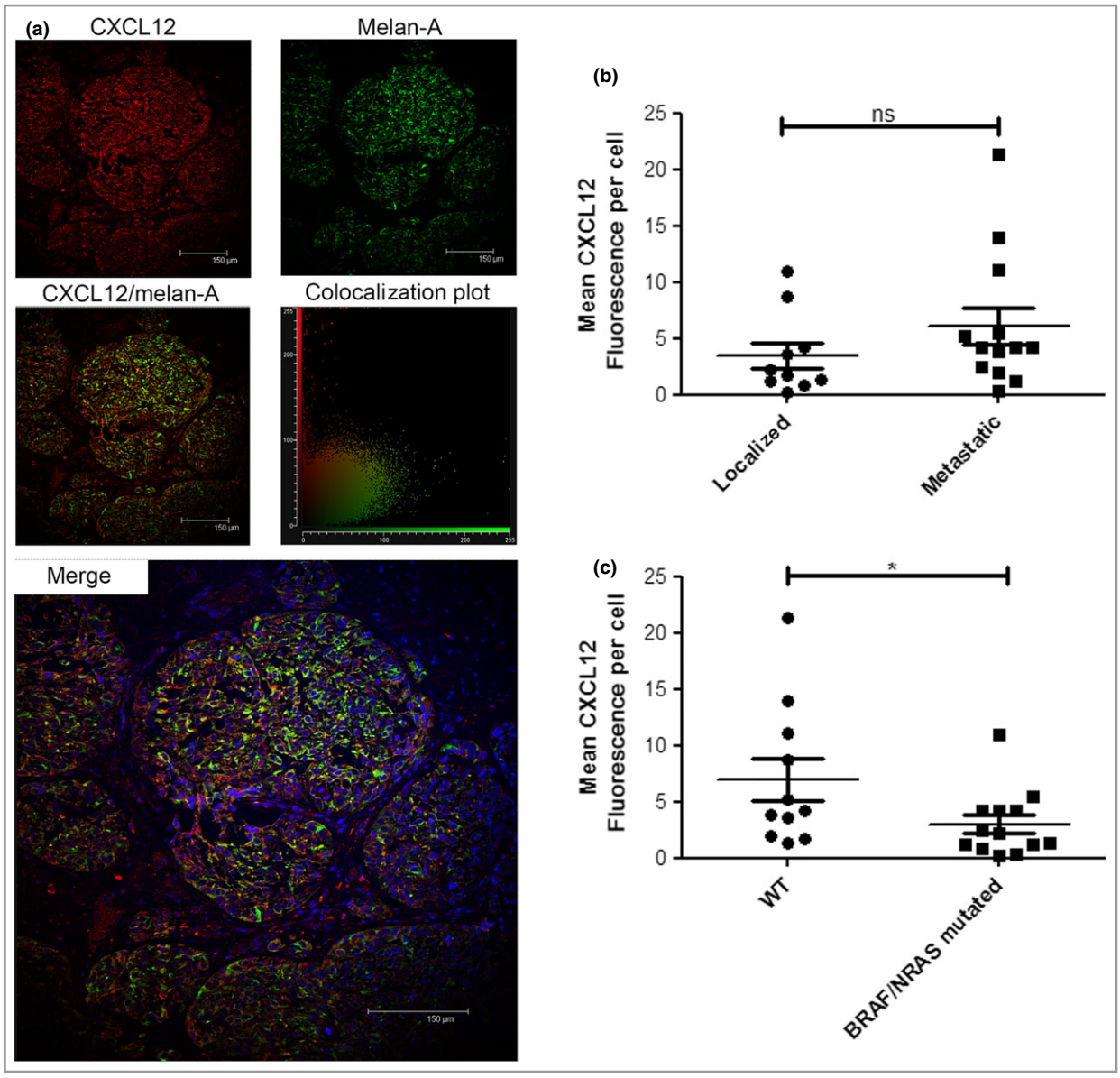

Fig 3. Downregulation of melanoma CXCL12 is associated with an aggressive BRAF/NRAS mutant phenotype. (a) Representative image of melan-A (green), CXCL12 (red) expression or nuclear 4′,6-diamidino-2-phenylindole (blue) staining in a primary American Joint Committee on Cancer (AJCC) stage III melanoma, including colocalization plot of melan-A and CXCL12 expression. Images were acquired with the null primary control set to no detectable fluorescence for each channel and by confocal microscopy with magnification ×20, scale bar = 150 µm. (b) Scatter graph representing the mean of 4 high-powered fields (HPF) of CXCL12 fluorescence/expression per melanoma cell in primary localized (eventual AJCC stage I/II disease) or metastatic melanomas (eventual AJCC stage III/IV). Horizontal bars represent the mean ± SD CXCL12 fluorescence per cell. Statistics were acquired by Student's t-test, $P = 0{\cdot}11$ (not significant). (c) Scatter graph representing the mean (4 HPF) of CXCL12 fluorescence per cell in all primary AJCC stage BRAF/NRAS wild-type (WT) or mutant melanomas. Horizontal bars represent the mean ± SD CXCL12 fluorescence per cell. Statistics acquired by Student's t-test, $P = 0{\cdot}032$.

mitosis or ulceration, tumour regression and early AJCC stage disease.[11] However, data from the current study clearly demonstrate that elevated CXCR4 expression in melanoma significantly correlates with a metastatic phenotype. Consistently with these findings, individuals with AJCC stage II primary cutaneous melanomas with high CXCR4 expression (> 50%) were threefold more likely to develop metastasis with concurrent reduced disease-free survival. Collectively these data define CXCR4 expression in primary melanomas as a novel prognostic biomarker for AJCC stage II disease, thus identifying a high-risk subgroup and enabling earlier therapeutic intervention.

Our data further show the expression of CXCL12 by melanoma cells and parenchymal cells within the cutaneous microenvironment, and additionally define an autocrine signalling mechanism whereby tumoral secretion of CXCL12 is

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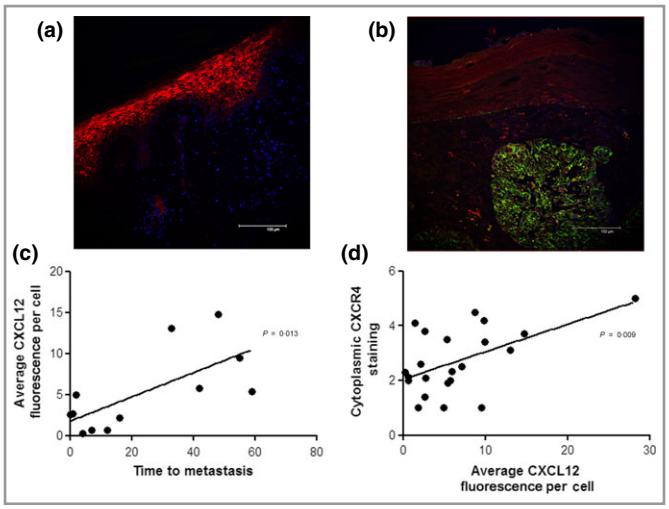

Fig 4. Increased CXCL12 in primary cutaneous melanoma epidermis correlates with increased time to metastasis. Representative images of blue 4′,6-diamidino-2-phenylindole staining or melan-A (green) and CXCL12 (red) expression depicted in the epidermis (a) adjacent to or (b) above an American Joint Committee on Cancer (AJCC) stage III primary melanoma tumour. Images were acquired with negative antibody control set to no detectable fluorescence for each channel. Images were acquired by confocal microscopy with magnification ×20, scale bar = 150 μm. (c) Scatter graph representing the mean of two high-powered fields (HPF) of CXCL12 fluorescence per cell in the adjacent epidermis of eventual AJCC stage III/IV melanomas vs. time to metastasis (months) after 7 years of follow-up. Statistics were acquired by Pearson correlation, P = 0·013. (d) Scatter graph representing the mean (2 HPF) CXCL12 fluorescence per cell in the adjacent epidermis of all AJCC stage melanomas vs. relative tumoral CXCR4 expression. Statistics were acquired by Pearson correlation, P = 0·009.

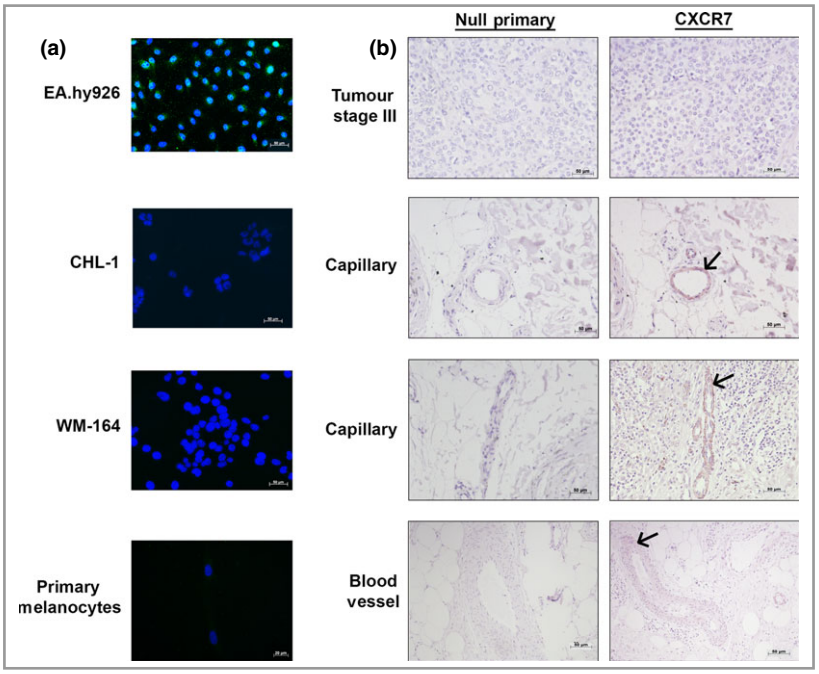

Fig 5. Expression of CXCR7 in cutaneous melanoma and the primary tumour microenvironment. (a) Representative images from three replicate experiments for the immunofluorescent expression of CXCR7 in EA.hy926 endothelial cells (positive control), metastatic melanoma cells CHL-1 or WM-164, and primary melanocytes. Images were acquired with negative antibody control set to no detectable fluorescence. Green depicts CXCR7 positivity and blue 4′,6-diamidino-2-phenylindole nuclear staining. Images were acquired by confocal microscopy with magnification ×20, scale bar = 50 μm. (b) Representative images of CXCR7 immunohistochemical expression in a cohort of primary cutaneous melanomas of differing American Joint Committee on Cancer stage. Images depict negative CXCR7 expression in the tumour bulk of an eventual stage III primary melanoma, and positive CXCR7 expression in capillary and blood vessels within the tumour microenvironment, or following staining with an isotype-negative primary antibody control. Images were acquired at ×20 magnification. The arrows depict positive CXCR7 staining in capillaries and larger blood vessels. Scale bar = 50 μm.

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able to promote activation of prosurvival MAPK cell signalling. Indeed, previous studies have illustrated CXCL12-induced activation of MAPK p44/42 (ERK1/2) and p38,[26] and in the context of our data they support a role for endogenous CXCL12 driving MAPK cell signalling in melanoma. To extend these findings *in situ*, our data further demonstrated that there was no significant difference in CXCL12 expression between melanomas that remained localized and those that subsequently metastasized. These results are corroborated by a previous study also revealing a lack of any significant correlation between CXCL12 expression and metastasis or morbidity.[10] This suggests a model whereby abundant endogenous CXCL12 within the primary site would act to retain CXCR4-positive tumour cells *in situ*, limiting their migration towards CXCL12-rich distant sites. Furthermore, CXCL12 expression by primary melanomas provides supporting evidence for autocrine CXCR4–CXCL12 cell signalling, which as shown in our *in vitro* studies promotes MAPK cell signalling, the likely consequence of which is sustained tumour maintenance and survival.

Previous studies have not provided evidence for any association of CXCL12 expression in melanoma with NRAS/BRAF mutational status.[11] Importantly, our data demonstrate significantly less CXCL12 expression in BRAF/NRAS mutant melanomas compared with wild-type tumours. This indicates, for the first time, an exciting potential mechanism for oncogenic BRAF/NRAS signalling in melanomagenesis, whereby oncogenic BRAF/NRAS signalling reduces CXCL12 expression by primary tumour cells. Similarly, previous studies suggest that the absence of CXCL12 expression correlates with poor clinical outcome.[11] Collectively, these and our data thus propose the absence/downregulation of CXCL12 expression by BRAF/NRAS primary melanomas, supporting the emergence of an aggressive disease phenotype and an enhanced ability for CXCR4-positive tumour cells to metastasize.

To date, the presence of CXCL12 in skin is documented[11]; however, the role of CXCL12 within the tumour microenvironment and its effect on the primary tumour have not been defined. Quantification of CXCL12 expression within the epidermis revealed a positive correlation with increased microenvironmental CXCL12 and increased time to metastasis. Similarly, increased tumoral cytoplasmic CXCR4 expression correlated with increased CXCL12 expression. These data are coherent as they suggest that tumoral CXCR4 is chronically activated by microenvironmental CXCL12, and is therefore internalized from the melanoma cell membrane. Importantly, chronic CXCR4 signalling by melanoma is supported by our *in vitro* observation of basal phospho-CXCR4 levels, phosphorylation of CXCR4 being characterized by ligand occupancy and a prerequisite of receptor internalization.[27] Moreover, our data suggest that a high level of CXCL12 within the epidermis adjacent to primary melanomas may act to retain CXCR4-positive tumour cells *in situ*, thereby promoting radial growth and delaying melanoma vertical invasion into the dermis and subsequent metastasis to distant sites.

CXCL12 interacts with its signalling receptor, CXCR4, and also the atypical/scavenging receptor CXCR7, recently redesignated ACKR3.[28] CXCR7 has a powerful capacity to scavenge CXCL12, and studies suggest that serum CXCL12 levels may be increased fivefold when CXCR7 is absent or inhibited.[29] Although CXCR7 is expressed at low levels within the primary cutaneous melanoma microenvironment, its observed expression by vascular endothelial cells supports previous observations of CXCR7 expression by endothelial cells in vasculature associated with breast and lung tumour.[30] This further supports the likelihood of CXCL12 sequestration, potentially altering chemokine gradients within the parenchyma and facilitating melanoma intravasation. Furthermore, although we did not observe any association between microenvironmental CXCR7 expression and melanoma progression, other studies suggest that high CXCR7 expression is often associated with periods of accelerated tumour growth, hypoxia and angiogenesis.[31]

Overall, our data highlight a previously undescribed role and cross-talk of the CXCR4–CXCR7–CXCL12 chemokine axis within the primary cutaneous melanoma microenvironment, and its intimate relationship with disease dissemination and clinical outcome. Moreover, the definition of high CXCR4 expression as a potential novel prognostic biomarker for AJCC stage II melanomas will enable earlier therapeutic intervention and improved patient outcomes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Video S1. Author video.

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